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(54) Refined detoxified
endotoxin (lipid A) and anti-tumour
compositions thereof

(57) A refined detoxified endotoxin
(RDE) product having no detectable 2-
keto-3-deoxyoctanoate, 350—475 n
moles/mg of phosphorus and 1700—
2000 n-moles/mg of fatty acids

(comprising crude lipid A) and
obtained from family
Enterobacteriaceae when combined
with cell wall skeleton (CWS), results
in a composition useful for the
treatment of cancerous tumors.
Methods of making RDE as well as
methods of using the combination of
RDE and CWS are referred to.

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The homogenized oil containing mixture is then combined with a detergent which may optionally be dissolved in a saline solution prior to mixing. The amount of detergent is typically between

- about 0.02 and 0.20 percent by volume and preferably between about 0.10 and 0.20 percent by volume based on the total volume of the composition. Any common detergent material may be used including Tween-80 and Arlacel (produced by the Atlas Chemical Company).

- The mixture resulting from the addition of detergent is then homogenized to form a suspension which has a high percentage of oil droplets coated with RDE and CWS as determined by observation under a microscope.

The following examples are for illustrative purposes only and are not intended to limit or in any way redefine the invention as claimed in the claims appended hereto.

20 Example 1

Preparation of crude detoxified endotoxin

- A 650 mg sample of a methanol-chloroform precipitate produced in accordance with the procedure of Chen, et al *J. Infect. Dis.* 128 543 (1973) was suspended in 150 ml of 0.1N HCl in a three necked round bottom flask fitted with a condenser, and immersed in a sonicator. After sonication, the glass apparatus was then lowered into an oil bath maintained at 120°C. which allowed the interior temperature of the flask to approach or exceed the boiling point of the solution. Superheating of the solution was minimized by fitting the flask with a capillary tube attached to a nitrogen gas source through one of the necks. A continuous flow of nitrogen was maintained throughout the hydrolysis procedure.

- Hydrolysis was continued for 30 minutes after which the solution was cooled in an ice bath, sonicated to disperse the solid material and distributed in corex tubes. The flask was washed with distilled water to remove all solid material adhering to the sides of the flask, and the wash was added to the suspension in the corex tubes. Centrifugation was carried out at 12,000 rpm for 80 minutes. The supernatant was decanted and discarded. The solid residue was resuspended in distilled water, sonicated until the suspension was well dispersed and recentrifuged. The centrifugation process was then repeated. The residue was taken up in distilled water, shell frozen and lyophilized yielding 382 mg of crude lipid A. 150 mg of this material were treated with cold (0°C.) acetone to remove fatty acids, sonicated, and filtered through a Whatman No. 1 gravity filtration apparatus at 5°C. 100 mg of crude detoxified endotoxin remained after drying.

Example 2

Preparation of crude detoxified endotoxin

- A 120 mg sample of MCP (methanol-chloroform precipitate) was suspended in 12 ml of absolute methanol, sonicated to disperse solid materials and distributed into 6 (1 x 10 cm) screw cap vials. 2 ml of 0.2N HCl were added to each

- tube and the resulting suspension was incubated in a boiling water bath for 45 minutes. After hydrolysis, the tubes were cooled in an ice water bath and centrifuged for about 10 minutes at 2500 rpm. The supernatant was decanted and 5 ml of a 2:1 chloroform/methanol mixture were added to the residue to effect dissolution. 2 ml of water were added per tube and the solution was mixed. The biphasic solution was recentrifuged at 2500 rpm for 10 minutes. The upper water phases was discarded at 1 ml of 4:1 chloroform/methanol mixture was added to each tube resulting in a clear solution. The solutions were pooled, and the solvent evaporated on a rotary evaporator. The residue was dried under vacuum and lyophilized to yield 45 mg of crude lipid A. 20 mg of this material were treated with cold (0°C) acetone, sonicated, and filtered through a Whatman No. 1 gravity filtration apparatus at 5°C. 13 mg of crude detoxified endotoxin remained after drying.

85 Example 3

Preparation of refined detoxified endotoxin

- 110 g LH-20-100 (25-100 micron particle size: Pharmacia) were combined with 600 ml of a 2:1 chloroform/methanol mixture which was permitted to stand for 30 minutes. The resulting slurry was added to a 25 x 1000 mm glass chromatography column (BRL Laboratories) fitted with pressure fittings. After packing was complete, the column was attached by means of Teflon pressure tubing to an ISCO Model 132 pump. 400 ml of a 4:1 chloroform/methanol mixture were pumped through the column at the rate of 3 ml/min. 100 mg of crude detoxified endotoxin prepared in accordance with Example 1 were applied to the column in 2.5 ml of a 4:1 chloroform/methanol mixture via a sample loop. The flow was reduced to 1 ml/min. and after 150 ml of eluant were collected, the effluent was connected to a fraction collector. 4 ml fractions were collected and refined detoxified endotoxin fractions were determined by thin layer chromatographic analysis of the fractions (E. Merck, 0.25 mm thick, chloroform/methanol/H₂O/NH₄OH (50:25:4:2) as eluant).

- The refined detoxified endotoxin fractions were combined and the solvent evaporated leaving 30 mg of refined detoxified endotoxin as a white powder.

Example 4

115 Preparation of refined detoxified endotoxin

- 33 g of DEAE-cellulose (Whatman DE-32) were suspended in 150 ml of glacial acetic acid and agitated gently for 10 minutes to obtain a slurry powder. The mixture was set aside overnight.

- The slurry was poured into a 25 x 400 mm column, allowed to settle with tapping, and excess acid was thereafter drained. The column was washed with 2000 ml of methanol followed by 200 ml of a 4:1 chloroform/methanol mixture. A 100 mg sample of crude detoxified endotoxin

produced in accordance with Example 1 was added to the column in 3 ml of a 4:1 chloroform/methanol mixture or an 80:20:1 mixture of chloroform, methanol and water.

- 5 column was eluted with 350 ml of a 99:1 methanol/water mixture. Using a linear gradient apparatus, the column was eluted with 2000 ml of a linear gradient starting with 100% methanol and ending with 0.2 M acetic acid in methanol.
- 10 The column was eluted at the rate of 6 ml/min. and 15 ml fractions were collected. Every other fraction was analyzed for total phosphorous content according to the procedure of Bartlett, G. R., *J. Biol Chem.* 234, 466—471 (1959). The
- 15 fractions were pooled and evaporated on a rotary evaporator to near dryness and taken up in 10 ml of a 2:1 chloroform/methanol mixture and 40 ml of 0.001 M acetic acid in a separatory funnel. The lower layer was separated, filtered through
- 20 Whatman No. 2 filter paper and evaporated to dryness to yield 19.2 mg of refined detoxified endotoxin.

Example 5

- Thirteen Strain 2 guinea pigs having Line-10 tumor growths of about 9 mm. were injected once with 0.4 ml of a sterile oil droplet emulsion, i.e., Drakeol 6 VR mineral oil (Pennsylvania Refining Company, Butler, Pennsylvania), containing 50 micrograms (RDE) and 50 micro-
- 30 grams (CWS) directly into the tumor tissue.

- At the end of a three month period, the animals were examined and in 12 of the 13 animals, total regression of the tumor growth has occurred. In a control experiment, 6 Strain 2 guinea pigs having
- 35 Line-10 tumor growths of about 9 mm. were injected once with 0.4 ml containing 50 micrograms of RDE alone. The injections were made directly into the tumor tissue. None of the 6 tumors showed any signs of regression after three
- 40 months.

Claims

1. A method of producing refined detoxified endotoxin having no detectable 2-keto-3-deoxyoctanoate, between about 350 and 475
- 45 nmoles/mg of phosphorus, and between about 1700 and 200 nmoles/mg of fatty acids which comprises:

- (a) hydrolyzing an endotoxin extract obtained from microorganisms of the Family Enterobacteriaceae with an acid such as hydrochloric acid, sulfuric acid, phosphoric acid, toluene sulfonic acid, or trichloroacetic acid;
- 50 (b) lyophilizing the hydrolyzed product to obtain crude lipid A;

- (c) treating crude lipid A with a first solvent such as acetone to dissolve fatty acids contained therein;

- (d) dissolving the resulting insoluble product in a second solvent such as methanol, chloroform, acetone, pyridine, ether and acetic acid, or a mixture of these solvents; and
- 60 (e) passing the resulting solution through a chromatographic column to obtain the desired product.

2. The method of Claim 1 wherein the reaction is conducted at a temperature between 90 and 130°C.

3. Refined detoxified endotoxin having no detectable 2-keto-3-deoxyoctanoate, between about 350 and 475 nmoles/mg of phosphorus and between about 1700 and 200 nmoles/mg of fatty acids.
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4. A therapeutic composition comprising a therapeutically effective amount of the refined detoxified endotoxin product according to Claim 3 in combination with cell wall skeleton and a pharmaceutically acceptable carrier.
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5. The composition of Claim 4 wherein the composition is a lyophilized form or in the form of an oil droplet emulsion.
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6. The composition of Claim 4 wherein the therapeutically effective amount of refined detoxified endotoxin is up to about 1500 micrograms per injection and the effective amount of cell wall skeleton is up to about 4500 micrograms per injection.
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7. A method of treating cancer in a warm-blooded animal comprising administering the composition of Claim 4 into said warm-blooded animal.
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8. The method of Claim 7 further comprising administering said composition containing between about 6.25 and 250 micrograms/ml of refined detoxified endotoxin and between about 125 and 750 micrograms/ml of cell wall skeleton, by injection directly into the tumour tissue of said warm blooded animal, said injections being made at intervals of at least one week.
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9. A method of treating cancer in humans comprising administering the composition of Claim 4 into said human, said composition containing between about 50 and 100 micrograms of each refined detoxified endotoxin and cell wall skeleton, and preferably between about 275 and 325 micrograms of each of the aforesaid materials.
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10. The method of Claim 9 further comprising administering by injection said composition containing between about 50 and 500 micrograms/ml of refined detoxified endotoxin and between about 50 and 500 micrograms/ml of cell wall skeleton, said composition being injected directly into the tumor tissue for up to fifteen injections and the injections being made at intervals of at least one week.
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